UNIVERSITI PUTRA MALAYSIA

PREVALENCE AND MOLECULAR CHARACTERIZATION OF VANCOMYCIN-RESISTANT ENTEROCOCCI ISOLATED FROM POULTRY, RAW VEGETABLES AND CLINICAL SOURCES

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By

OOI WAI LING

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

August 2003

DEDICATIONS

To my beloved parents, brothers, sisters, relatives and friends. Also to those who have taught me and still continue in teaching me of different angles of life. Also to those who have sincerely provided invaluable assistance, guidance, advice, moral support and encouragement through out the whole project including the writing out part.

OOI WAI LING 2003

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Doctor of Philosophy

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Chairman: Associate Professor Son Radu, Ph.D.

Faculty: Food Science and Biotechnology

In this study, vancomycin-resistant *Enterococcus* (VRE) were isolated from 120, 60, 180, 450, 850, and 850 with chicken samples (CS), whole chicken (W), hospital clinical samples (HCS), environmental chicken faecal samples (CFS), ulam (U) and vegetables samples, respectively. All these samples were obtained mainly from wet markets and areas around Sri Serdang, Seri Kembangan and Kuala Lumpur. Vancomycin-resistant enterococci isolated from these samples were characterized phenotypically and genotypically. The prevalence of VRE isolated are as follow: HCS (14/180, 7.8%) with 1 isolate as *E. faecalis* (1/14, 7.1%) and 13 as *E. faecium* (13/14, 92.9%); CFS (27/450, 6%) with 24 isolates as *E. raffinosus* (24/27, 88.9%) and 3 as *E. faecium* (3/27, 11.1%); W (8/60, 13.3%) with 1 isolate as *E. faecalis* (1/8, 12.5%), 2 as *E. gallinarum* (3/8, 37.5%), and 4 as *E. faecium* (4/8, 50.0%); CS (16/120, 13.3%) with 2 isolates identified as *E. gallinarum* (2/16, 12.5%), 3 as *E. faecalis* (3/16, 18.8%) and 11 as *E. raffinosus* (11/16, 84.6%); U

(8/850, 0.9%) with 3 isolates as *E. faecium* (3/8, 37.5%) and 5 as *E. raffinosus* (5/8, 62.5%); V (8/850, 0.9%) with 5 as *E. faecium* (5/8, 62.5%) and 3 as *E. gallinarum* (3/8, 37.5%). The MAR index for VRE isolates examined ranged from 0.07-0.93 with *E. raffinosus* having the highest ARI value. Among the 14 antibiotics tested, the highest prevalence of resistance was against kanamycin, streptomycin, erythromycin, bacitracin, tetracycline, and vancomycin (100%); cefuroxime and chloramphenicol (93%), penicillin G (89%), gentamicin (52%), while the lowest resistance was observed for ampicillin (40%), ceftriaxone (34%), rifampicin (10%) and teicoplanin (0%). The VRE isolates generated various plasmid profiles with sizes ranging from 1.7-35.8 MDa. Most isolates were found devoid of plasmid. The multiplex PCR (M-PCR) displayed 3 identified isolates habouring the *van*A gene (3.7%), whereas, in single PCR, 42 isolates harboured the *van*A gene (51.85%). Both M-PCR and single PCR identified 1 isolate (V13) habouring *van*B gene, whilst, 16 VRE isolates isolated from CFS, V and U samples were positive for *van*C*1* gene. No *van*C2,3, *van*D, *van*E and *van*G genes were detected among the isolates. Three random primers, GEN 1-50-03 (5′-CTT GAG TGG A-3′), GEN 1-50-05 (5′-TCC TCAAGA C-3′), and GEN 1-50-08 (5′-GAG ATG ACG A-3′) used in RAPD-PCR analysis produced bands with molecular size ranging from 0.25 kb to 3.0 kb. PFGE analysis using *Sma*I (5′-CCC↓GGG-3′) produced high discrimination fragment patterns among all the VRE isolates examined. Based on the data obtained from the fragment profiles in both RAPD-PCR and PFGE analysis, relationship among the isolates was determined by phylogenetic software (RAPDistance v.1.04). In the immunological evaluation, passive haemagglutination assay (PHA) was used to check immunogenecity. BM4147 gave the highest PHA value followed by CFS66, V583, and BM4174 in the 5 VRE cells treatment groups. The antibody titres in PHA,

demonstrated a relative correlation between the immunogenicity and the VRE cells antigenic properties on the cell walls. The application of PCR (*van* genes) and immunological evaluation methods enable the retrieval of information on both pathogeneicity and immunogeneicity of VRE isolates examined. Our results in the study demonstrates that the plasmid profiling, M-PCR, and single PCR techniques are able to act as useful analysis tools for rapid and reliable typing and identification of VRE; whilst, the RAPD and PFGE analysis are useful discrimination tool to differentiate the VRE isolates examined.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PREVALEN DAN PENCIRIAN SECARA MOLEKULAR ENTEROCOCCI KERINTANGAN VANKOMISIN YANG DIPENCILKAN DARI SUMBER-SUMBER TERNAKAN AYAM, SAYURAN MENTAH DAN KLINIKAL

Oleh

OOI WAI LING

Ogos 2003

Pengerusi: Profesor Madya Son Radu, Ph.D.

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Di dalam kajian ini, *Enterococcus kerintangan* vankomisin (VRE) telah dipencilkan daripada 120, 60, 180, 450, 850, dan 850 dengan sampel ayam kisar (CS), sample ayam basuhan (W), klinikal (HCS), sample persekitaran dari tinja ayam (CFS), ulam (U) dan sampel sayuran. Semua sample ini telah diperolehi daripada pasar-pasar dan kawasan di sekitar Sri Serdang, Seri Kembangan dan Kuala Lumpur. Enterococci kerintangan vankomisin yang telah dipencilkan dicirikan selanjutnya secara fenotipik dan genotipik. Prevalen VRE yang dipencilkan adalah seperti berikut: HCS (14/180, 7.8%) dengan satu isolate sebagai *E. faecalis* (1/14, 7.1%), manakala tiga belas sebagai *E. faecium* (13/14, 92.9%); CFS (27/450, 6%), 24 isolate dikenalpasti sebagai *E. raffinosus* (24/27, 88.9%) dan tiga sebagai *E. faecium* (3/27, 11.1%); W (8/60, 13.3%) dengan satu isolate dikenalpasti sebagai *E. faecalis* (1/8, 12.5%), 2 sebagai *E. gallinarum* (3/8, 37.5%), dan 4 sebagai *E. faecium* (4/8, 50.0%); CS (16/120, 13.3%) dengan 2 sebagai *E. gallinarum* (2/16, 12.5%), 3 sebagai *E. faecalis* (3/16, 18.8%) dan 11 sebagai *E. raffinosus* (11/16, 84.6%); U (8/850, 0.9%) dengan 3 isolate dikenalpasti sebagai *E. faecium* (3/8, 37.5%) dan 5

sebagai *E. raffinosus* (5/8, 62.5%); V (8/850, 0.9%), dengan 5 dikenalpasti sebagai *E. faecium* (5/8, 62.5%) dan 3 sebagai *E. gallinarum* (3/8, 37.5%). Index MAR bagi isolate VRE mempunyai nilai julat 0.07-0.93 manakala *E. raffinosus* adalah spesis yang memberikan nilai ARI tertinggi. Daripada 14 antibiotik yang dikaji, prevalen kerintangan tertinggi dalam isolate VRE kajian adalah terhadap kanamisin, streptomisin, erithromisin, basitrasin, tetrasiklin dan vankomisin (100%); sefuroxime dan kloramfenikol (93%), penisiillin G (89%), gentamisin (52%), manakala kerintangan terendah untuk ampisillin (40%), seftriaxon (34%), rifampisin (10%) dan teikoplanin (0%). Isolate VRE memberikan profil plasmid dengan julat saiz dari 1.7-35.8 MDa. Kebanyakan isolate menunjukkan ketidakhadiran plasmid. Analisis multiplex PCR (M-PCR) memaparkan tiga isolate yang dikenalpasti menunjukkan kehadiran gen *van*A (3.7%) berbanding 42 isolate yang positif untuk gen *van*A dalam PCR tunggal (51.85%). Kedua-dua analisis M-PCR dan PCR tunggal mengenalpasti 1 isolate yang positif kepada gen *van*B (V13), manakala 16 isolate daripada CFS, V dan U adalah positif kepada gen *van*C1. Tiada sebarang gen yang dikesan untuk gen *van*C2,3, *van*D, *van*E dan *van*G untuk kesemua isolate yang dikaji. Tiga primer rawak, GEN 1-50-03 (5′-CTT GAG TGG A-3′), GEN 1-50-05 (5′-TCC TCAAGA C-3′), dan GEN 1-50-08 (5′-GAG ATG ACG A-3′) yang digunakan dalam analisis RAPD menghasilkan fragmen yang bersaiz molekul antara 0.25 kb-3.0 kb. Analisis yang menggunakan enzim pemotong *Sma*I (5′-CCC↓GGG-3′), menghasilkan fragmen corak yang berdiskriminasi tinggi di antara semua isolate VRE yang dikaji. Berdasarkan maklumat yang diperolehi daripada profil fragmen di dalam kedua-dua analisis RAPD-PCR dan PFGE, hubungan di antara isolate telah dikenalpastikan dengan menggunakan cakera ringan untuk analisis filogenetik (RAPDistance v.104) Di dalam Kajian immunology, kaedah hemagglutinasi pasif (PHA) telah digunakan

untuk mengkaji immunogenisiti. BM4147 memberikan nilai PHA yang tertinggi berikutan dengan CFS66, V583, dan BM4174 di dalam lima kumpulan rawatan sel VRE. Titer antibodi memaparkan korelasi relatif di antara immunogenisiti dan kesan antigenik pada permukaan dinding sel VRE. Aplikasi kaedah PCR (gen *van*) dan kaedah kajian immunology membolehkan maklumat tentang patogenisiti dan imunogenisiti diperolehi untuk kajian isolate VRE. Keputusan kita turut menunjukkan kaedah profil plasmid, M-PCR, dan PCR tunggal adalah berkemampuan digunakan sebagai alat penganalisaan yang cepat, berkesan dan bersesuaian bagi pencirian dan pengenalan VRE; manakala analisis dengan RAPD-PCR dan PFGE amat berguna sebagai alat perbezaaan untuk membezakan isolate VRE yang dikaji.

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Finally, words just can't express what is beholding in my heart, please accept my sincere thankfulness and gratitude for everything.

I certify that an Examination Committee met on 29th August, 2003 to conduct the final examination of Ooi Wai Ling on her Doctor of Philosophy thesis entitled "Prevalence and Molecular Characterization of Vancomycin-Resistant Enterococci Isolated from Poultry, Raw Vegetables and Clinical Sources" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committees are as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

(OOI WAI LING)

Date: 26/10/2003

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Abbreviations

A	adenine or adenosine
AP-PCR	arbitrarily primed-polymerase chain reaction
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
Amp	ampicillin
B	bacitracin
bp	basepair
BSA	bovine Serum Albumin
C	chloramphenicol
ccc	covalently closed circular
cm	centimetre
Cro	ceftriaxone
Cxm	cefuroxime
Da	dalton
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanine triphosphate
dTTP	deoxythymidine triphosphate
dH_2O	distilled water
DNA	deoxyribonucleic acid
dNTPs	deoxy nucleotide triphosphate (PCR nucleotide mix containing dATP, dTTP, dGTP and dCTP)
E	erythromycin

E.coli	*Escherichia coli*
e.g.	for example
EDTA	ethylenediamine tetraacetic acid
EtBr	ethidium bromide
g	gram
g	gravity
G	guanine
Gm	gentamicin
h	hour
i.e.	that is
I	intermediate
ID	identification number
K	kanamycin
kb	kilobase
KDa	kiloDalton
kg	kilogram
LB	Luria-Bertani
M	molar, or molarity, moles of solute per litre of solution
MDa	megaDalton
mg	miligram
MHA	Mueller Hinton Agar
min	minutes
ml	mililitre
mm	millimetre
mM	miliMolar

µg	microgram
µl	microlitre
mol	mole
Na	sodium
NaCl	sodium chloride
NaOH	sodium hydroxide
P	penicillin
%	percentage
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
R	resistant
RAPD	random amplified polymorphic DNA
Rd	rifampicin
RE	restriction enzyme
REA	restriction enzyme analysis
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNase	ribonuclease
rpm	rotation per minute
s	sensitive
S	streptomycin
sdH_2O	sterile distilled water
SDS	sodium dodecyl suphate
Taq	*Thermus aquaticus* DNA (polymerase)
TBE	tris borate EDTA electrophoresis buffer

TE	tris EDTA buffer
Te	tetracycline
Tec	teicoplanin
Tris	tris (hydroxylmethyl) methylamine
UV	ultraviolet
V	volts
Va	vancomycin
v/v	volume per volume
w/v	weight per volume